

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
25 April 2002 (25.04.2002)

PCT

(10) International Publication Number
WO 02/33066 A1

(51) International Patent Classification⁷: C12N 13/00 //
15/89

CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EC, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(21) International Application Number: PCT/SE01/02301

(22) International Filing Date: 19 October 2001 (19.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0003841-4 20 October 2000 (20.10.2000) SE

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(81) Designated States (national): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA,

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— of inventorship (Rule 4.17(iv)) for US only

Published:

— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/33066 A1

(54) Title: A COMBINED ELECTROPORATION AND MICROINJECTION METHOD FOR THE PENETRATION OF LIPID BILAYER MEMBRANES

(57) Abstract: Disclosed is a method for penetration of lipid bilayer membranes in order to insert at the tip of a hollow needle-shaped object, such as a micropipet-, into a container formed of a lipid bilayer membrane, wherein said container is placed between said needle-shaped object, with the tip of said needle-shaped object placed in contact with said container in such a way that it applies a mechanical force to the lipid membrane of said container, thus mechanically straining it, and a second electrode, whereupon a transient electric pulse of 1-to- 10^3 V/cm is applied between the electrodes, resulting in a focused electrical field over said container which induces a dielectric breakdown of the lipid bilayer causing the needle-shaped object to penetrate the container. Disclosed is also an electroinjection method based on the above method, wherein substances are introduced through the needle-shaped object and into the container after penetration of the needle-shaped object.

**A COMBINED ELECTROPORATION AND MICROINJECTION METHOD
FOR THE PENETRATION OF LIPID BILAYER MEMBRANES**

Field of the invention

The present invention relates to a method for the penetration of lipid bilayer membranes in order to insert tips of needle-shaped objects into lipid membrane enclosed containers, such as cells and liposomes. The invention also 5 relates to a method for the injection of a substance into a lipid bilayer container, such as a cell, or more precisely to a microinjection method utilizing the concept of electromechanical destabilization for the efficient loading of substances, such as biopolymers, colloidal particles, and other biologically relevant molecules, into single cell-sized lipid bilayer containers.

10

Background of the invention

Today, there is a growing interest in inserting microelectrodes, microcapillaries and micropipet-tip-sensors into single cells. There is also a growing interest in incorporating sub-micron-sized sensing, sampling, and signal-amplifying particles, as well as large biopolymers into single cells and liposomes. Several ultrasensitive detection and sensing methods are based directly or indirectly on the use of colloidal particles. Examples include quantum dot bioconjugate sensors^{1,2}, the family of Probes Encapsulated By Biologically Localized Embedding (PEBBLE) sensors,³ and silver (Ag) and gold (Au) colloids for use in Surface Enhanced Raman Spectroscopy (SERS) measurements⁴⁻⁶. One of the main limitations for practically using these techniques is the difficulty of noninvasive and quantitative introduction of colloidal particles into the cellular interior⁷. Furthermore, it would be attractive to direct the introduction of particles into specific subcellular compartments such as the cytosol, nucleus, or even organelles of individual cells.

25 GUVs are cell-sized liposomes composed of a single lipid bilayer with an entrapped aqueous compartment⁸. Such liposomes are attractive to use as ultra-small reaction containers in which the reaction under study is confined and separated from the external medium. As such they can be used for studies 30 of biochemical reaction dynamics in compartments mimicking a natural intra-cellular-intraorganellar environment⁹⁻¹².

For use as reaction containers, it is necessary to load vesicles with reactants, including biopolymers like DNA and colloid particles or organelles (synthetic or naturally derived). Loading of liposomes can, in principle, be performed by adding the particles during the preparation of the vesicles, since 5 they upon formation trap a part of the medium in which they are formed. The trapping efficiency for small liposomes is, however, limited even for low-molecular-weight compounds and, entrapment of larger structures such as colloids, is of very low probability^{13,14}.

Another approach for liposome-loading is to introduce the materials into 10 preformed vesicles by using micromanipulation-based techniques developed for loading of single cells. One such technique that is feasible to use is the microinjection technique¹⁵.

By using microneedles made out of pulled glass capillaries with outer tip-diameters in the range of 200-500 nm, it is possible to penetrate the membrane wall of a liposome, or cell, and eject controlled volumes of a desired reagent inside the vesicle¹⁶. Injection volumes are typically in the picoliter to attoliter range and controlled by regulation of injection-time and injection-pressure. The pressure is usually generated by utilization of pressurized-air or oil-hydraulic systems.

20 All microinjection techniques are based on mechanical permeabilization of lipid membranes. When a mechanical point-load is applied, e.g. by a capillary, onto the membrane of a liposome or cell, the membrane is forced to stretch and the isotropic membrane tension, working in the plane of the membrane, is increased. At sufficiently high membrane tension, the structural integrity of the liposome, or cell, is momentarily lost as holes are formed in the membrane, releasing internal fluid in order to counteract the increase in membrane tension. This membrane rupture occurs at the site of the highest mechanical load, which is the loci where the point-load is applied, thus allowing 25 the insertion of a microinjection capillary into the interior of the liposome or cell.

Whereas microinjection works well with certain cell-types and multilamellar liposomes, there are a few drawbacks to the microinjection techniques with unilamellar vesicles and many cell types. Lipid membrane bilayers are, typically, very elastic and the absence of internal supporting structures in 30 unilamellar liposomes make them very difficult to penetrate by mechanical means. The outer diameter of a tip suitable for injection into thin-walled lipo-

- somes and smaller cells is about 200 nm, and the inner diameter is typically in the range of only 100 nm^{16,17}. Such tips are very fragile and extremely difficult to view in a light microscope, making positioning difficult. The main drawback of using small inner-diameter injection tips is, however, the requirement of
- 5 using ultrapure injection liquids in order to prevent clogging, limiting injection species to solutions of low- and medium-molecular-weight compounds. Micro-injection techniques are considered to be relatively invasive due to the large mechanical forces applied, inducing permanent membrane damage and even lysis of cells and liposomes.
- 10 An alternative approach to single-liposome or single-cell loading is to use microelectroporation¹⁸. This technique is based on the theory of electro-permeabilization. When exposing a liposome, or cell, to an electrical field, a potential drop is generated across the membrane. At sufficiently high field strength, the critical transmembrane potential V_c , of the membrane is exceeded, and small pores will form in the liposomal/cellular membrane due to dielectric membrane breakdown. The transmembrane potential V_m , at different loci on the membrane of a spherical vesicle during exposure to a homogeneous electric field of duration t , can be calculated from

$$V_m = 1.5 r_s E \cos \alpha (1 - \exp(-t/\tau))$$

- 20 where E is the electric field strength, r_s is the radius of the sphere, α is the angle in relation to the direction of the electric field, and τ is the capacitive-resistive time constant. Pore formation will occur at spherical coordinates exposed to the largest potential shift, which is at the poles facing the electrodes. Typical value for V_c for a cell-sized vesicle is ~1V, and the corresponding
- 25 electric field strength needed for exceeding the critical transmembrane potential V_c , is in the range of 1-10 kV/cm.

In microelectroporation, the analyte to be encapsulated is added to the exterior solution of the liposomes, or cells, and an electrical field is then applied locally, using microelectrodes. The amount of analyte that enters the

30 vesicle is dependent on the analyte concentration gradient, membrane potential, duration of the applied field, and diffusion rate of the analyte¹⁹. Drawbacks to the electroporation technique are difficulties of quantitative loading, and loading of structures of sizes larger than the effective pore-diameter, which for electroporated erythrocytes is in the range of 1-to-240 nm^{20,21}. To improve quantitative loading, controlled amounts of analytes can be introduced via a small micropipette tip inserted into a hole pre-formed by electroporation (as

described, for example, in JP 8322548). This approach, however, presents a number of disadvantages, including the need to apply a fairly strong electric field ($\sim 1\text{ V}$) to form a hole for tip insertion.

By combining electroporation and the application of a mechanical force onto a membrane vesicle, the strength of the applied electrical field needed for membrane permeabilization may be substantially reduced²⁵. This phenomenon is sometimes referred to as electro-mechanical destabilization. It has been shown that electrical fields established over lipid bilayer membranes imposes an electrocompressive mechanical stress σ_e , acting on the lipid membrane. This force works normal to the plane of the membrane and leads to a decrease in membrane thickness. If assuming that a lipid membrane behaves as a capacitor, then the electro-compressive force is proportional to the voltage drop V , over the membrane and thus to the strength of the applied electric field

$$15 \quad \sigma_e = \frac{1}{2} \epsilon \epsilon_0 \cdot \left(\frac{V}{h_e} \right)$$

where ϵ , is the relative dielectric constant and ϵ_0 , is the permitivity and h_e , is the dielectric thickness of the membrane. The differential overall mechanical work dW , done on the lipid membrane is then simply the sum of the electro-compressive stress σ_e , and the isotropic membrane tension \bar{T} , controlled by the amount of mechanical strain applied to the membrane

$$dW = \left[\bar{T} + \frac{1}{2} \epsilon \epsilon_0 \left(\frac{V}{h_e} \right) \cdot h \right] dA$$

25 where h is the overall thickness of the lipid bilayer membrane, and dA is the change in membrane area. Consequently, when a mechanical strain is applied to a membrane vesicle, the trans-membrane potential needed to achieve permeabilization can be significantly reduced. Therefore this approach for membrane permeabilization may be even less invasive than electroporation since lower 30 electric fields can be used, minimizing the risk of unwanted electrochemical reactions at the membrane surface of a cell or a liposome.

Summary of the invention

The present invention relates to a novel approach for inserting micropipet tips or any other cylindrical or hollow needle-shaped objects such as micro-electrodes into containers formed of lipid bilayer membranes, such as cells and

liposomes. The basic idea is to destabilize a mechanically strained lipid membrane container with electric pulses, facilitating the penetration of a micropipet.

The invention also relates to a method for introducing substances, such as large-molecular-weight compounds as well as colloid particles into containers formed of lipid bilayer membranes, such as GUVs, cells, and other similar membrane enclosed structures, by applying the concept of electro-mechanical membrane destabilization to a micropipet-assisted microinjection technique. The unique advantage of such an arrangement arises from the combination of the high degree of spatial- and volume-control of microinjection and the efficient and non-invasive membrane permeabilization of electromechanical destabilization, thereby permitting quantitative introduction of analytes, and biologically relevant molecules and particles including colloids, into unilamellar liposomes and cells.

More specifically, the invention relates to a method for the penetration of a container formed of or surrounded by at least one lipid bilayer membrane in order to insert the tip(s) of at least one hollow needle-shaped object into said container, wherein said container is placed between said at least one needle-shaped object, such as an electrolyte-filled micropipet, equipped with a first electrode, which preferably is an internal electrode, and a second electrode, wherein the tip of said at least one needle-shaped object is placed in contact with said container in such way that said tip of said at least one needle-shaped object applies a mechanical force to the lipid bilayer membrane of said container, thus mechanically straining said container, whereupon a transient electric pulse of 1-to- 10^3 V/cm is applied between said first electrode and said second electrode, resulting in a focused electric field over said container, said electrical field inducing dielectric breakdown of the lipid bilayer causing the tip of said at least one needle-shaped object to penetrate the membrane of said container. Said at least one needle-shaped object is hollow and preferably constructed from an insulating material and is filled with an electrically conducting solution or with dispersion of a substance.

Preferably said first electrode is an internal electrode, i.e. located inside said hollow needle-shaped object. More preferably, the electrode is connected to said at least one needle-shaped object, which in this case is filled with an electrolyte, in which the electrode is placed. This embodiment has several advantages. For example, no electrochemical reaction is present at the tip of said at least one needle-shaped object since the electrode is located at a distance

from the tip of said at least one needle-shaped object. The presence of such an electrochemical reaction would otherwise negatively affect the container, especially for cases when the container is a cell since the health and survivability of the cell then would deteriorate.

5 When the tip of the needle-shape object, such as a micropipet tip, has penetrated the container any of the solution or dispersion contained in the micropipet may be injected into the container by a variety of methods including pressure-induced and electro-osmotic flow, whereupon the micropipet may be removed, and this is used as a basis for the microinjection or electroinjection
10 method according to the invention.

The electroinjection technique described here has several distinct advantages when compared to traditional stab-microinjection protocols. First of all, the technique is less invasive. Since the membrane is electrically destabilized, less mechanical force is needed for penetrating the lipid membrane with a micropipet or any other needle-shaped object of micro-dimensions. Consequently,
15 there is less movement of the injection capillary when located inside a liposome or cell. Such movements may induce severe cell trauma caused by damages to the cellular matrix.

If compared to electroporation protocols, much lower transmembrane
20 potentials are needed to achieve membrane destabilization, often the generated transmembrane potential is only a few mV (which is further explained in the examples below) two to three orders of magnitude smaller than the V_m used in electroporation. Much lower electric fields translates into less electrically induced trauma to the cell, as well as minimizing the risk of unwanted electrochemical reactions at the membrane surface of a cell or a liposome. The usage
25 of very low electric field strengths is, beside the fact that the membrane is under mechanical strain, also an effect of the highly focused electric field that is used. When a voltage is applied between the first and the second electrode, the non-conducting material of the needle-shaped object, such as a micropipet, directs the entire established electrical field through the tip-end opening of the pipet. Consequently, the part of the lipid membrane that is in contact with the tip-end of the needle-shaped object is exposed to the entire electrical field. The coordinates of maximum electro-compressive force thus spatially coincides
30 with the loci where maximum mechanical force is applied.

35 The presented method for inserting the tips of needle-shape object, such as micropipets, into containers such as liposomes and cells is much more effi-

cient than standard stab-microinjection protocols. As a consequence, large diameter micropipets can be used, allowing injections of large structures into unilamellar vesicles, as well as cells. From this, several possibilities arise. One attractive application is the quantitative introduction of nanosensors¹⁻³ or col-
5 loids, for SERS measurements⁴⁻⁶ into cells for detection of molecules or for probing of intracellular structures. Another application is introduction of these particles into liposomal reaction containers. Such a procedure would allow studies of complex biochemical reactions where the formation of several products and intermediates simultaneously could be monitored. By incorporation of
10 organelles (naturally or synthetically derived), or even bacteria into unilamellar vesicles it is possible to create highly advanced cell models. This is very attractive for studies of, for example, complex biochemical signaling systems that translocate between different intracellular compartments.

The electroinjection technique described here can be performed with
15 very high success rates and allows sequential injection of multiple reagents into single liposomes and cells without noticeable leakage. Initiation of complex biochemical reactions inside the confines of a liposome or cell is therefore feasible. This also makes it possible to perform ultra-small-scale derivatization chemistry inside a liposome, or cell, for analyte labeling prior to microchemical separations.
20

If combined with the ultra-thin injection needles used for conventional microinjections¹⁷, the method according to the present invention is a powerful technique for introduction of low- and medium-molecular-weight compounds into smaller cells or even organelles. This is due to the efficient membrane
25 penetrative capacity of the electroinjection technique.

Another application is in the field of so called chip-array injections. Since the technique here presented is highly efficient in terms of membrane penetration capacity, it is feasible to construct an array injector system where a plurality of injection needles-tips are clustered together, arrayed, allowing parallel injection of a large number of cells simultaneously.
30

Detailed description of the invention

As stated above, the present invention relates to a method for the penetration of lipid bilayer membranes in order to insert at least one tip of at least
35 one needle-shaped object, such as a micropipet tip, into lipid membrane containers. More specifically the method is used for the penetration of a container

constituted of at least one lipid bilayer, wherein said container first is placed between a hollow electrolyte filled, non-conducting needle-shape object, such as a micropipet, equipped with a first electrode, preferably an internal electrode, i.e. an electrode located inside said needle-shaped object, with the tip of
5 said at least one needle-shaped object placed in contact with said container in such a way that said needle-shaped object applies a mechanical force to the lipid membrane of said container, thus mechanically straining said container, and a second electrode, whereupon, by the use of a low-voltage supply, a transient electric pulse of 1-to- 10^3 V/cm is applied between said first electrode and
10 said second electrode, thus establishing an electric field between said first and said second electrode, passing through the tip-end of the said needle-shaped object, resulting in a highly focused electrical field over the membrane part of said container that is in contact with said needle-shaped object , said electrical field inducing a local dielectric breakdown of the part of the mechanically
15 strained lipid bilayer that is in contact with said needle-shaped object, causing the tip of the needle-shaped object to penetrate the membrane of the container.

Below, the term micropipet is used instead of needle-shaped object, however what is stated for the micropipet is valid also for other needle-shaped objects, such as other cylindrical or hollow needle-shaped objects of appropriate size, such as capillaries, ultra-thin injection needles, and microelectrodes.
20 Below, the term also encompasses arrays of such needle-shaped objects, which, for example, may be mounted on a chip. Furthermore, the micropipet shall be filled with a conductive medium to allow electrical contact between the first electrode and the second electrode.

25 Said hollow, non-conducting micropipet, is filled with an electrically conducting solution or dispersion of a substance. When the micropipet has penetrated the container, any of the solution or dispersion of a substance contained in the micropipet may be injected into the container, after which the micropipet may be removed. In addition, the micropipet and microelectrode
30 makes a pair of tweezers allowing for the manipulation and subsequent injection into the free-floating cells and liposomes.

Said substance contained in the micropipet and to be injected into a cell or liposome may be, for example, a low or medium molecular weight substance, such as a dye, a biopolymer, such a DNA, RNA or a protein, a colloidal particle, such as a colloidal bead, a nanosensor, an organelle, or a bacterium.
35 The expression "low or medium molecular weight substance" relates to a sub-

stance with a molecular weight of up to a few kDa, such as up to 3 kDa. The substance is preferably injected into the container in the form of a solution or dispersion. A small volume, typically $50\text{-to-}500 \times 10^{-15}$ l, of the solution or dispersion is injected into the cell or other unilamellar container.

5 Said container shall be constituted of or surrounded by at least one lipid bilayer membrane. It may, for example, be a liposome, a vesicle, an organelle, a cell, a multilamellar liposome (MLV) or a giant unilamellar vesicle (GUV). The method according to the invention is particularly interesting for giant unilamellar vesicles and cells. The size of said unilamellar containers shall be
10 organelle or cell-sized, i.e. 0.1-to- 10^3 μm in diameter.

Said micropipet should be prepared from a nonconductive material in order to contain and focus the applied electric field through the tip-end opening (the end closest to the container) of said micropipet, and may, for example, be a glass-, a quartz-, or a plastic-micropipet. The micropipet shall preferably
15 have an outer diameter at the tip of 10 nm-to-100 μm , and an inner diameter, i.e. the diameter of the hollow space inside the micropipet, of 0.05-to-95 μm . Furthermore, the micropipet shall be filled with a conductive medium to allow electrical contact between the first electrode and the second electrode. The micropipet shall be equipped with a first electrode, preferably an internal, highly
20 conductive electrode, such as a Pt-, Ag-, Au-, or carbon fiber-electrode, however an electrode of any suitable conductive material may be used. The tip of the first electrode, located inside said micropipet, should be placed at a distance, preferably 0,5-to-1 cm, from the tip of the micropipet in order to prevent direct contact between said first electrode and the lipid bilayer membrane of
25 the container, thus protecting the membrane from electrochemically generated reactive species and/or gas bubbles that may form on the first electrode surface.

Said second electrode may be constituted of any suitable conductive material. It may, for example, be a carbon fiber-, a metal-, or a glass-micro-electrode. The second electrode shall preferably have a diameter in the end placed next to the container of approximately 1-to- 10^3 μm . The first and second electrodes may be similar or different. It is also feasible to exchange the second electrode for a ground bath-type electrode.

The voltage pulse used to obtain the highly focused electric field between the first and the second electrode, inducing the dielectric breakdown of the lipid bilayer shall be a transient electric pulse of a field strength of 1-to- 10^3

V/cm. Preferably a transient 0,01-to-10 ms, rectangular waveform dc-voltage pulse of 10-to-60 V/cm is used but other pulseforms as well as ac-voltage can be used.

Once the micropipet tip is inserted into the lipid membrane container,
5 the micropipet can be used for several different purposes. For example, the micropipet can be used for sampling of intracontainer substances, the micropipet may also be a sensor such as a fiber optic- or electrochemical-microsensor used for intracontainer measurements or a microelectrode. Finally the micropipet can be used for injection of a substance into the container.

10 When a substance is to be introduced into the container this can be done in many different ways. It is, for example, possible to use a technique based on electrophoresis, electroendoosmosis, gravity flow, or microinjection with the aid of compressed air or oil, or a thermo-sensitive expansion medium.

Once a substance is introduced into the lipid bilayer container, the container can be used for many different purposes. For example, when the substance introduced is colloidal particles, such as Ag or Au colloids, quantum dot bioconjugate sensors^{1,2}, or PEBBLE sensors³ the container may be used in conjunction with an ultrasensitive detection or sensing method, such as SERS or quantitative fluorescence measurements, for detection of specific substances.
15

20 Another interesting application is the introduction of material into specific subcellular compartments such as the cytosol, nucleus, or even organelles of individual cells.

Brief description of the drawings

25 Below reference is made to the accompanying drawings on which:

Fig. 1 is a schematic drawing of a capillary holder, consisting of a main body (II) equipped with a Pt wire electrode attached to a connector pin (III) and an entrance for the microinjector outlet (I). The injection tips (VI) are held in place by two rubber O-rings (IV) and a screwcap (V).

30 Fig. 2 illustrates electroinjection of fluorescein into a giant unilamellar liposome. (A) is a Differential Interference Contrast (DIC) image showing two multilamellar liposomes with two adjacent unilamellar liposomes settled on the coverslip surface. The microelectrode and injection capillary were positioned in an opposing fashion close to the target liposome. (B) illustrates how a mechanical pressure was applied on the liposome by moving the injection tip towards the microelectrode, forcing the liposome into a kidney-like shape. (C)
35

illustrates how the membrane was permeabilized and the liposome was sled onto the injection tip and a fluorescein solution was injected into the liposome. (D) shows how the injection tip and counter electrode were removed from the liposome. (E) is a fluorescence image of the liposomes after injection. The 5 liposome injected with fluorescein is exhibiting strong fluorescence while the other liposomes were unaffected. The contour lines of the unilamellar liposomes were digitally enhanced.

Fig. 3 illustrates injection of biopolymer and colloid particles into GUVs. The figure shows fluorescence images of unilamellar liposomes injected with highly concentrated solutions of (A) 30 nm fluorescent latex spheres, (B) small (100 nm) SBL-liposomes (50 µg/ml), stained with DiO, and (C) YOYO-1 labeled T7 DNA (5 ng/ml).

Fig. 4 illustrates electroinjection of YOYO-1-stained T7 phage DNA into PC-12 cells. (A) and (B) show brightfield and fluorescence images of cells injected with fluorescent DNA into the cytosol. In (C) and (D) DNA is injected preferentially into the nucleus of the cell and the cytosol only shows faint fluorescence.

Fig. 5 shows a unilamellar liposome as a reaction container for the intercalation reaction between T2 DNA and YOYO-1. (A) shows a unilamellar protrusion from a multilamellar liposome used as target. (B) shows injection of a solution containing the T2 DNA into the liposome. (C) is a fluorescence image of the DNA-injected vesicle displaying no fluorescence. (D) shows how the injection capillary was withdrawn and replaced by a thinner capillary loaded with YOYO-1 for a second injection. (E) is a fluorescence image after 20 incubation revealing the presence of fluorescent YOYO-1-intercalated DNA molecules inside the liposome. Brownian motion of micrometer-sized structures could be observed in the microscope, strongly suggesting that the fluorescence originated mainly from YOYO-intercalated DNA. The YOYO-1 dye, 25 however, also had affinity for the lipid membranes as shown by the strong fluorescence originating from the multilamellar liposome.

Examples

MATERIALS AND METHODS

Chemicals

35 FM 1-43, DiO, YOYO-1 and FluoSpheres (30-nm- and 200-nm-diameter) were from Molecular probes. Fluorescein (GC-Grade), T2 DNA

(168 000 bp²²), T7 DNA (39936 bp²³), L- α -phosphatidylcholine (type II-S), potassium phosphate (>98%) and Trizma base (>99.9%) were purchased from SIGMA. Chloroform, EDTA (titriplex III), magnesium sulfate and potassium dihydrogen phosphate (all pro analysi) were obtained from MERCK. Glycerol 5 (>99.5%) from J. T. Baker and deionized water from a Milli-Q system (Millipore) was used.

Formation of small unilamellar vesicles (SUVs)

An acetone-purified asolectin preparation dissolved in chloroform was used²⁴. When preparing SUVs, the lipids were diluted with chloroform to a 10 lipid concentration of 10 mg/ml. For a standard preparation, 300 μ l of this solution was transferred to a round-bottomed flask. The solvent was removed on a rotary evaporator for about 6 h at room temperature. A thin completely dry lipid film had then formed on the walls of the flask. To this film, PBS buffer (Trizma base 5mM, K₃PO₄ 30 mM, KH₂PO₄ 30 mM, MgSO₄ 1 mM, EDTA 0.5 mM, pH 7.8.) containing 1% v/v glycerol, was carefully added to a lipid concentration of 1 mg/ml. The lipid film was allowed to swell overnight at 4°C. Finally, the sample was sonicated in a bath-type sonicator filled with ice water. A total sonication time of about 10 min was normally required before the entire lipid film dissolved and a whitish opalescent mixture was formed. The SUV-20 suspension was stored at 4°C and was stable for several days.

Formation of GUVs

The formation of GUVs was performed in a two-step procedure; dehydration of the lipid dispersion followed by rehydration.

For dehydration, a small volume (5 μ l) of SUV-suspension was carefully placed on a borosilicate coverslip and placed in a vacuum dessicator at 4°C. When the sample was completely dry (no sign of "fluidness" in microscope), the dehydration was terminated and the sample was allowed to reach room temperature before rehydration.

The dry sample was first rehydrated with 5 μ l buffer. After 3-5 min the 30 sample was further diluted with buffer, this was done very carefully to minimize turbulence in the sample. All rehydration liquids were at room temperature.

Micromanipulation and electroinjection

All injection experiments were performed on an inverted microscope 35 (Leica DM IRB, Wetzlar, Germany) equipped with a Leica PL Fluotar 40x objective and a water hydraulic micromanipulation system (high graduation ma-

nipulator: Narishige MWH-3, Tokyo, coarse manipulator: Narishige MC-35A, Tokyo).

Fluorescence imaging was achieved by sending the output of an Ar⁺-laser (Spectra-Physics 2025-05, 488nm) through a 488-nm line interference filter followed by a spinning disc to break the coherence and scatter the laser light. The laser light was collected by a lens and was sent through a fluorescein filter (Leica I-3) into the objective to excite the fluorescent dyes. The fluorescence was collected by the objective and detected by a three-chip color CCD camera (Hamamatsu, Kista, Sweden) and recorded on VHS (Panasonic S-VHS AG-5700). Digital image editing was performed using an Argus-20 system (Hamamatsu, Kista, Sweden) and Adobe Photoshop graphic software.

The electroinjections were controlled by a microinjection system (Eppendorf Transjector 5246, Hamburg, Germany) and a pulse generator (Digitimer Stimulator DS9A, Welwyn Garden City, U.K.) connected to the injection capillary.

For translation of liposomes to different locations during the experiments, carbon fiber microelectrodes (ProCFE, Axon Instruments, Foster City, CA) controlled by the micromanipulation system were used. By simply pushing the vesicles with the microelectrodes, they detached from the surface and adhered to the electrode tips and could be moved over long distances to a desired target. With this technique it was also possible to detach unilamellar protrusion-vesicles that adhered to multilamellar liposomes.

Preparation of injection tips

Injection tips were prepared from borosilicate capillaries (length: 10 cm, o.d.: 1 mm, i.d.: 0.78 mm; Clark Electromedical Instruments, Reading, UK) that were carefully flame-forged in the back ends in order to make entrance into the capillary holder easier. The capillaries were flushed with a stream of nitrogen gas before use. The tips were pulled on a CO₂-laser puller instrument (Model P-2000, Sutter instrument Co., Novato, CA). The outer diameter of the injection tips varied between 0.5-2.5 µm. To avoid contamination, tips were pulled immediately before use.

RESULT AND DISCUSSION

Microinjection procedures

The injection tips were back-filled with a medium of choice and mounted onto an in-house constructed pipet holder shown schematically in fig-

ure 1. The main purpose of the capillary holder is to secure the injection tip and to act as an interface between the microinjection system and the pulse generator. Basically the device is a standard patch-clamp pipet holder fitted to the outlet of a microinjection system. The main body of the pipet holder (II), in
5 this example, was constructed from Plexiglass, and equipped with a Pt-wire electrode connected to a low-voltage pulse generator via a connector pin (III). It also comprises an entrance for the microinjector outlet (I). The injection tips (VI) are firmly held in place by two rubber O-rings (IV) secured by a Delrin screwcap (V). The pipet-holder was mounted on the micromanipulation system
10 described above. A carbon fiber microelectrode with a tip diameter of 5 μm was used as counter electrode. After selecting an appropriate GUV or cell, the injection tip and the microelectrode were positioned in an opposing fashion, in close contact with the vesicle at an angle of 10-30° and 150-170° with respect to the object plane (see Fig. 2). By careful positioning of the electrodes it was
15 possible to trap free-floating vesicles and subsequently perform injections. By applying a mechanical pressure in terms of moving the injection tip towards the microelectrode, forcing the vesicle into a kidney-like shape (fig. 2B), it was possible to penetrate the membrane by applying the electric field (a rectangular dc-voltage pulse 40 V/cm, 3 ms). When permeabilized, the vesicle sled onto
20 the injection tip and regained its spherical form (fig 2C). In this mode, controlled volumes of materials contained in the micropipet could be injected into the liposome. In figure 2C, a 25- μM solution of fluorescein was injected into a single liposome. Injection volumes were controlled by the microinjection system (injection pressure: 250-1000 hPa, time: 0.1-1.5 s). Typically, a volume of
25 50-to-500 fL was injected into liposomes with a diameter of 10-to-20 μm . Injection volumes for cells were kept as small as possible in order to prevent cell trauma. After completed injection, the tip was withdrawn from the interior of the vesicle without noticeable signs of vesicle damage (fig. 2D) or leakage (fig.
2E).

30 GUVs as well as cells were permeabilized in a single-pulse mode, by applying one or several transient rectangular dc-voltage pulses with pulse durations of 1-10 ms. The electric field strength was typically in the range of 10-40 V/cm. The membrane voltage V_m , at different loci on the membrane of a vesicle during exposure to a homogeneous electric field of duration t , can be
35 calculated from

$$V_m = 1.5 r_s E \cos \alpha (1 - \exp(-t/\tau))$$

where E is the electric field strength, r_s is the radius of the sphere, α is the angle in relation to the direction of the electric field, and τ is the capacitive-resistive time constant. Even though this equation does not exactly match the conditions for the electroinjection technique, it can be used for roughly estimating the transmembrane potential generated. Assuming that a voltage pulse of 40 V/cm is applied at right angles over a spherical membrane container with a radius of 10 μm , a transmembrane potential of only 60 mV is generated. Clearly, such a small voltage drop across the membrane does not generate sufficient electro-compressive stress to achieve membrane permeabilization. Since the primary electrode is located inside the injection capillary, the coordinates of electric destabilization spatially coincides with the loci where maximum mechanical force is applied. This electro-mechanical permeabilization proved to be a powerful technique for penetration of lipid membranes, allowing the use of coarse micropipet tips.

15

Results

When using this procedure, we could inject reagents into single cells and GUVs with diameters of 5-to-25 μm using micropipet tips with an outer diameter of about 2 μm , or up to 3 μm . Therefore, injection into larger cells can be readily accomplished. Capillaries this coarse also have sufficiently large inner diameters for injection of larger structures and colloid particles at high concentrations into vesicles or cells. This is illustrated by the fact that YOYO-1-labeled T7 phage DNA molecules ($R_G=0.56 \mu\text{m}$), 30nm latex spheres as well as 100-nm-diameter SUVs were injected into unilamellar vesicles (see Fig. 3).

Single PC12 cells were successfully injected with fluorescein (data not shown) as well as T7 phage DNA labeled with YOYO-1 (as shown in fig. 4). Moreover, preferential but not exclusive injection of materials could be performed into the cytoplasm (fig. 4 B), and nucleus (fig. 4 D) of single cells. In both experiments the fluorescence is concentrated locally at the site of injection, indicating that diffusion of DNA-dye complex through the cell was restricted. This shows that the technique here presented would allow directed delivery of, for example, drugs, genetic material (such as DNA and RNA), proteins, dyes, and particles into specific compartments of a cell.

Since the electroinjection technique described here can be performed with very high success rates, it may be a powerful tool for initiation of chemical reactions inside vesicles and cells. This is illustrated by the experiment

shown in figure 5. By performing two consecutive injections of reagents into a single vesicle, an intercalation reaction between T2 phage DNA ($R_G=1.1\text{ }\mu\text{m}$) and YOYO-1 was initiated. (A) A unilamellar protrusion from a multilamellar liposome settled on the coverslip was selected as target. (B) First a solution containing the T2 DNA (1 ng/ml) was injected into the vesicle using a micro-pipet tip with an outer diameter of 2 μm (40 V/cm, 4ms). (C) Fluorescence image of the DNA-injected vesicle displayed no fluorescence. (D) The injection capillary was withdrawn and replaced by a thinner capillary with an outer diameter of 1 μm loaded with YOYO-1 (50 μM), and a second injection was performed (20 V/cm, 4ms). (E) Fluorescence imaging after 10 min incubation revealed the presence of fluorescent YOYO-1-intercalated DNA molecules inside the vesicle. Brownian motion of micrometer-sized structures could be observed in the microscope, strongly suggesting that the fluorescence originated mainly from YOYO-intercalated DNA. This experiment illustrates, except from the fact that chemical reactions can be initiated this way, also that it is possible to sequentially inject multiple reagents into a single vesicle without noticeable leakage. Initiation of complex biochemical reactions inside the confines of a liposome or cell is therefore feasible.

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CLAIMS

1. A method for penetration of lipid bilayer membranes in order to insert at least one tip of at least one hollow needle-shaped object into a container constituted by at least one lipid bilayer, wherein said container is placed between said at least one needle-shaped object, which is equipped with a first electrode, and a second electrode, wherein said at least one tip of said at least one needle-shaped object is placed in contact with said container in such a way that said at least one tip applies a mechanical force to the lipid bilayer of the container thus mechanically straining the container, whereupon a transient electric pulse of a field strength of 1-to- 10^3 V /cm is applied between said first electrode and said second electrode, resulting in a focused electrical field over said container resulting in a dielectric breakdown of the lipid bilayer causing said at least one tip of said at least one needle-shape object to penetrate the lipid bilayer membrane of the container.
5
10
2. A method according to claim 1, wherein said first electrode is located inside said needle-shaped object.
15
3. A method according to claim 1 or 2, wherein said needle-shaped object is a micropipet.
4. A method according to claim 3, wherein said micropipet is a glass micropipet.
5. A method according to claim 3, wherein said micropipet is a quartz micropipet.
20
6. A method according to claim 3, wherein said micropipet is a plastic micropipet.
7. A method according to claim 1 or 2, wherein said needle-shaped object is a microelectrode, which thus also constitutes said first electrode.
25
8. A method according to claim 1 or 2, wherein said needle-shaped object is a capillary.
9. A method according to any one of the claims 1 - 8, wherein the diameter of said container is 0.1-to- 10^3 μm .
- 30 10. A method according to any one of the claims 1 - 9, wherein the outer diameter of the tip of said needle-shape object is 10 nm-to-100 μm , provided that it is always smaller than the diameter of the container.
11. A method according to any one of the claims 1 - 10, wherein the inner diameter of the tip of said needle-shape object is from 50 nm up to

95 µm, provided that it is always smaller than the diameter of the container and always smaller than the outer diameter of the tip of said needle-shaped object.

12. A microinjection method comprising performing the method for penetration of lipid bilayer membranes according to any one of the claims 5 1 - 11, wherein a solution or dispersion of at least one substance is delivered through said needle-shape object contains and into said container once the tip of the needle-shape object has penetrated the container.

13. A method according to claim 12, wherein said substance is a low or medium molecular weight substance.

10 14. A method according to claim 12, wherein said substance is a bio-polymer.

15 15. A method according to claim 12, wherein said substance is a colloidal particle.

16. A method according to claim 12, wherein said substance is a nanosensor.

17. A method according to claim 12, wherein said substance is an organelle.

18. A method according to claim 12, wherein said substance is a bacterium.

20 19. A method according to claim 12, wherein said substance is a cell.

20. A method according to any one of the claims 1 - 19, wherein said micropipet is constituted by an array of several needle-shaped objects.

21. A method according to any one of the claims 1 - 20, wherein said first electrode is a Pt-electrode.

25 22. A method according to any one of the claims 1 - 20, wherein said first electrode is an Ag-electrode.

23. A method according to any one of the claims 1 - 20, wherein said first electrode is an Au-electrode.

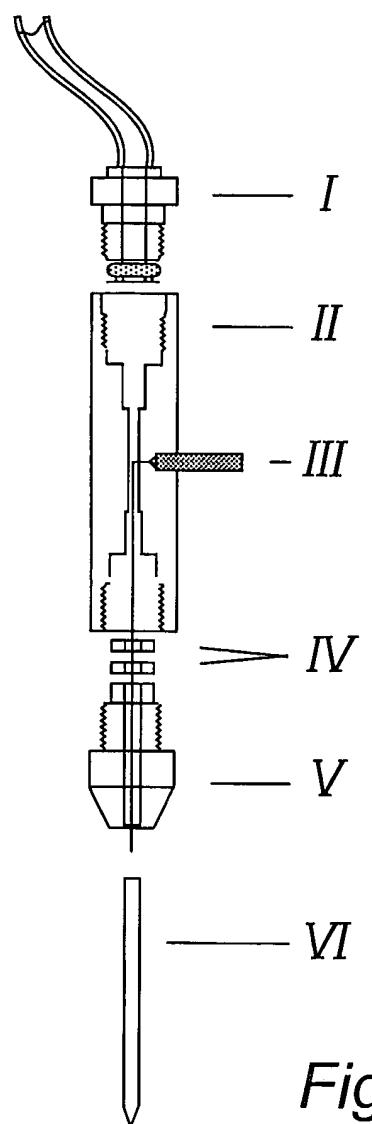
30 24. A method according to any one of the claims 1 - 20, wherein said first electrode is a carbon fiber-electrode.

25. A method according to any one of the claims 1 - 24, wherein the diameter of the second electrode, in the end close to the container, is approximately 1-to- 10^3 µm.

35 26. A method according to any one of the claims 1 - 25, wherein said second electrode is a carbon fiber electrode.

27. A method according to any one of the claims 1 - 25, wherein said second electrode is a metal electrode.
28. A method according to any one of the claims 1 - 25, wherein said second electrode is a glass micro-electrode.
- 5 29. A method according to any one of the claims 1 - 28, wherein said container is a liposome.
30. A method according to any one of the claims 1 - 28, wherein said container is a vesicle.
- 10 31. A method according to any one of the claims 1 - 28, wherein said container is an organelle.
32. A method according to any one of the claims 1 - 28, wherein said container is a cell.
33. A method according to any one of the claims 1 - 28, wherein said container is a so-called giant unilamellar vesicle (GUV).
- 15 34. A method according to any one of the claims 1 - 28, wherein said container is a so-called multilamellar vesicle (MLV).
35. A method according to any one of the claims 12 - 34, which is repeated once or several times in order to insert different substances into said container.
- 20 36. A container treated with a method according to any one of the claims 1 - 35.
37. Use of a container according to claim 36 in the detection of specific molecules.
- 25 38. Use of a container according to claim 36 in the probing of intracellular structures.
39. Use of a container according to claim 36 in the study of biochemical reactions.
40. Use of a container according to claim 36 as a container for chemical derivatization in a miniaturized separation technique.
- 30 41. Use of a container according to claim 36 in a biological computer.

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*Fig. 1*

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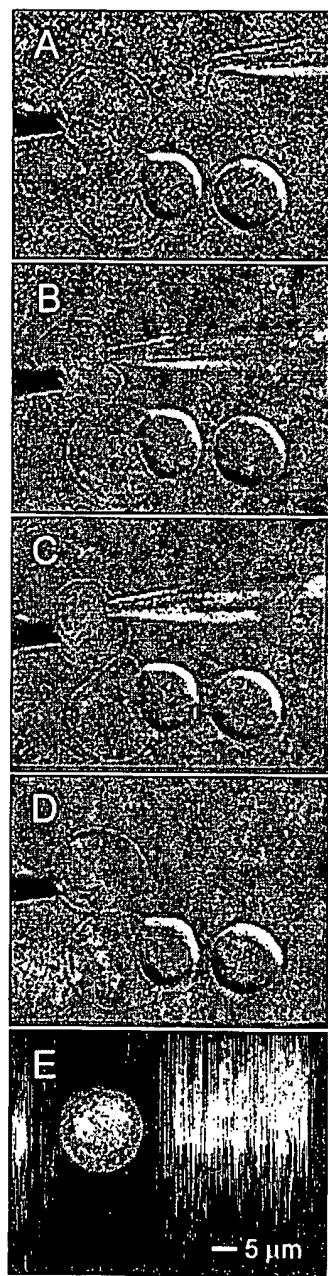


Fig. 2

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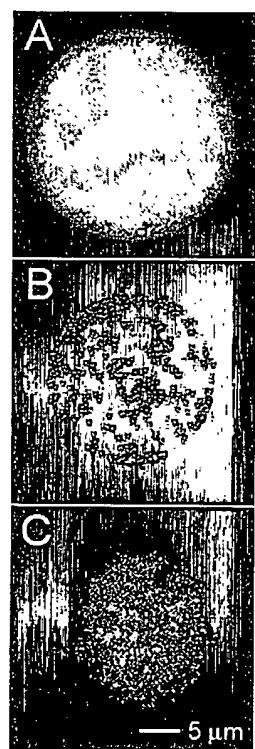


Fig. 3

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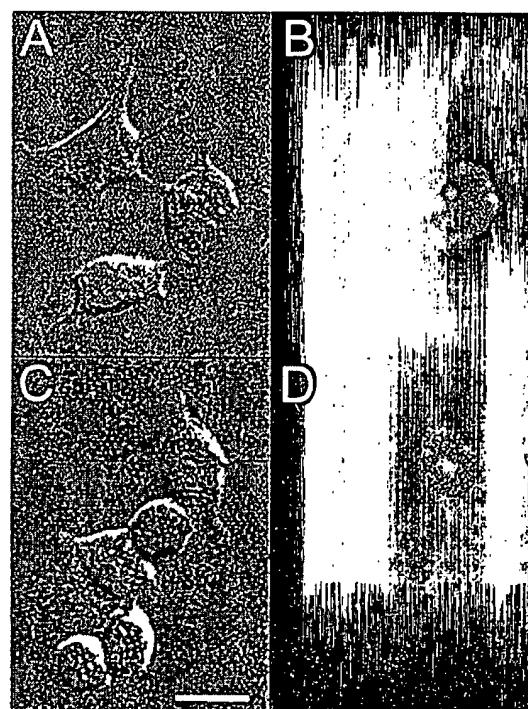


Fig. 4

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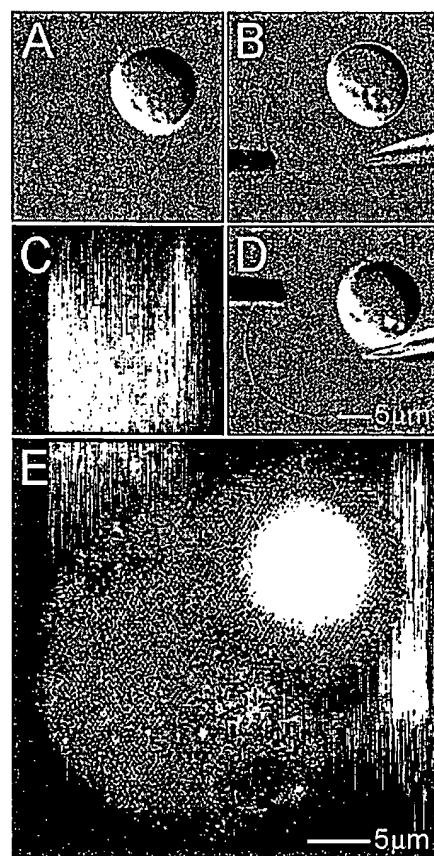


Fig. 5

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INTERNATIONAL SEARCH REPORT

Interr... at application No.

PCT/SE 01/02301

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 13/00 // C12N 15/89

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, A61N, C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WPI/Derwent's abstract, Accession Number 1997-081068, week 9708, ABSTRACT OF JP, 8322548 (NIKON CORP), 10 December 1996 (10.12.96)	1-35
X	--	36-41
A	WO 0034434 A1 (ACACIA BIOSCIENCES, INC.), 15 June 2000 (15.06.00)	1-35
X	--	36-41

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Date of the actual completion of the international search

Date of mailing of the international search report

20 February 2002

21-02-2002

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 01/02301

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9924110 A1 (A+ SCIENCE INVEST AB), 20 May 1999 (20.05.99)	1-35
X	--	36-41
A	Developmental Biology, Volume 233, 2001, M. Swartz et al: "Sparking New Frontiers: Using in Vivo Electroporation for Genetic Manipulations", pages 13-21	1-35
X	--	36-41
P,X	Anal. Chem., Volume 72, 2000, Mattias Karlsson et al: "Electroinjection of Colloid Particles and Biopolymers into Single Unilamellar Liposomes and Cells for Bioanalytical Applications", pages 5857-5862	1-41
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Information on patent family members

International application No.

28/01/02

PCT/SE 01/02301

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 0034434 A1	15/06/00	AU 2150300 A		26/06/00
		JP 2000101623 A		07/04/00
WO 9924110 A1	20/05/99	AU 1059399 A		31/05/99
		CN 1280513 T		17/01/01
		EP 1028778 A		23/08/00
		JP 2001522666 T		20/11/01
		SE 9704076 D		00/00/00